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TITLE	(54):	Method for Detecting Cytokeratins
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ABSTRACT (57): This invention relates to a method for detecting cytokeratins, involving the following steps:

a) Taking and preparing a body sample,
b) Isolating mRNA from the body sample,
c) Converting the mRNA into cDNA through reverse transcription,
d) Amplifying the cDNA using a PCR with primers exhibiting a higher affinity to the cDNA of cytokeratins than to processed cytokeratin pseudogenes, and
e) Detecting the amplified cDNA,
The invention also relates to primers for executing the method along with a kit.

Specification

The invention relates to a method for detecting cytokeratin 18 (CK18) via polymerase chain reaction along with primers useable for this purpose and a detection kit.

Cytokeratins are a group of keratin-like filaments, of which in particular cytokeratin 18 (hereinafter called CK18) has become very important, since it can be used to detect tumors originating from epithelial cells. This detection is based on immunological methods, for example, in which antibodies to CK18 are used. Such methods are not very sensitive, however. On the other hand, CK18 is also detected on a nucleic acid level. To this end, CK18-mRNA is converted into cDNA through reverse transcription, and the latter is amplified in a polymerase chain reaction (PCR) and then detected. However, this type of method, called RT-PCR, often yields false-positive results, which makes its specificity unsatisfactory.

Neumaier et al., Gene 159 (1995), pp. 43-47, describe the diagnosis of micrometastases via the amplification of tissue-specific genes. This is done, for example, by amplifying CK-18 or CEA in a polymerase chain reaction.

WO 96/17080 relates to the detection of tumors via the expression of cytokeratin 20.

The object of this invention is hence to provide a method with which CK18 can be sensitively and specifically detected.

This object is achieved according to the invention by the subject matter as set forth in the claims.

Therefore, the subject of this invention is a method for detecting CK18 that involves the following steps:

a) Taking and preparing a body sample,

b) Isolating mRNA from the body sample,

c) Converting the mRNA into cDNA through reverse transcription,

d) Amplifying the cDNA using a PCR with special primers to be specified below, which exhibit a higher affinity to the cDNA of CK18 than to processed CK18 pseudogenes, and

e) Detecting the amplified cDNA.

This invention is based on the knowledge of the applicant that the false-positive results in a conventional RT-PCR for CK18 are caused by processed CK18 pseudogenes. These are present as contaminants in the prepared mRNA of a body sample. The processed CK18 pseudogenes are very homologous to the cDNA of CK18, and are therefore concurrently amplified in a conventional RT-PCR method.

The applicant has isolated processed CK18 pseudogenes. To this end, it isolated DNA from the human genome and subjected it to a PCR, making use of primers that are also suitable for amplifying CK18-cDNA. The amplified pseudogene-DNA was cloned and sequenced. Three of the amplified pseudogene-DNA's are depicted on Fig. 1 in comparison to CK18-cDNA. On March 7, 1997, the amplified pseudogene-DNA's were filed with the DSM under DSM 11448, DSM 11447 and DSM 11446 as clone 6, clone 5 and clone 1, respectively. They also constitute a subject of this invention.

In a method according to the invention, the difference between the cDNA of CK18 and its processed pseudogenes is used to construct primers for a PCR, resulting in a selective amplification of the cDNA of cytokeratins relative to processed cytokeratin pseudogenes.

A method according to the invention involves taking and preparing a body sample. Suitable body samples include smears, the punctate fluid or biopsy of an organ, e.g., bone marrow, but also blood, sputum, urine, stool, fluid, bile, lymphatic liquid or gastrointestinal secretion, wherein a bone marrow biopsy or blood is preferred.

The body sample is taken and prepared in the usual manner. The mRNA is also isolated from the body sample based on a conventional method. It is favorable to use the guanidinium-thiocyanate-phenol-chloroform method (compare Chomczynski, P and Sacchi, N. Anal. Biochem. 162, (1987), 156-1691.

The obtained mRNA is subjected to reverse transcription, wherein use is made of conventional primers, e.g., "random hexamers" from Pharmacia. A conventional reverse transcriptase can also be used, preferably an MMLV reverse transcriptase (e.g., Superskript II, Gibco BRL). The conditions and buffers of the reverse transcription are as recommended by the manufacturer.

The obtained cDNA is subjected to a PCR, wherein it is favorable to use a commercially available Taq polymerase (e.g., Gibco BRL). The primers used have a higher affinity to the cDNA of cytokeratins than to processed cytokeratin pseudogenes. These primers can be obtained using conventional methods. It is favorable to proceed as follows: Primers for areas of the cDNA of cytokeratins, in particular CK18, are constructed in which the processed cytokeratin pseudogenes exhibit differences (compare Fig. 2). Processed cytokeratin pseudogenes or their sequences are provided via conventional methods. By way of example, reference is made to the provision of processed CK18 pseudogenes. The obtained primers are tested for their affinity to the cDNA of cytokeratins or processed cytokeratin pseudogenes. This can be done in conventional tests. For example, the primers are used in a PCR that incorporates the cDNA of cytokeratins or processed cytokeratin pseudogenes as genomic DNA. The PCR can be executed under conventional conditions, yielding primers exhibiting a higher affinity to the cDNA of cytokeratins than to processed cytokeratin pseudogenes. The conditions for the PCR, such as time, temperature, pH, buffer, can also be varied, so that the optimal conditions are determined for each primer pair under which it has the highest affinity to the cDNA of cytokeratins or the lowest affinity to processed cytokeratin pseudogenes.

Primers that can be used in a method according to the invention constitute a subject of this invention.

Primers according to invention in terms of the cDNA of CK18 are: 5'-TGCTCACCACACAGTCTGAT-3' (X), 5'-CACTTTGCCATCCACTAGCC-3' (Y), 5'-TGGAGGACCGCTACGCCCTA-3' (X') AND 5'-CCAAGGCATCACCAAGACTA-3' (Y'). Fig. 1 indicates the position of the primers. Primers X and X' can be used as "upper" primers, while Y and Y' can be used as "lower" primers. Primer pairs X and Y as well as X' and Y' are especially preferred.

Conventional conditions can be observed for executing the PCR in a method according to the invention. The PCR reaction batch preferably consists of 50 mM KCl, 10 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatins, 0.25 mM of each dNTP, 0.4-0.8 µM primer and 5 units of Taq polymerase per 100 µl.

The PCR advantageously involves the following per cycle: Denaturing the DNA at 90 to 100°C for 40 to 60 seconds, in particular at 95°C for 50 seconds, along with hybridizing the primers at 40 to 60°C for 20 to 40 seconds, in particular at 54°C for 30 seconds.

In addition, the PCR reaction can involve denaturing at 90 to 100°C for 1 to 2 minutes, in particular at 95°C for 80 seconds, followed by extension at 65 to 75°C for 30 to 90 seconds, in particular at 72°C for 1 minute.

An expert can easily determine the number of cycles in the PCR. It can depend on the tissue from which the mRNA originated. The number of cycles preferably measures 20 to 100, wherein 30 to 70 cycles are especially preferred for mRNA from blood, and 40 to 80 cycles for mRNA from bone marrow.

It can be favorable to use two different primer pairs in the PCR, wherein the second primer pair consists of "nested" primers, thereby amplifying a smaller DNA segment inside the amplified DNA segment of the fist PCR. This type of PCR reaction preferably encompasses the following:

First PCR: X and Y primers, approx. 40 cycles at 90 to 100°C for 40 to 60 seconds, in particular 95°C for 50 seconds, and 40 to 60°C for 20 to 40 seconds, in particular 54°C for 30 seconds.

An aliquot of the first PCR is diluted with the batch for the second PCR, e.g., 1 : 10-1 : 20.

Second PCR: X' and Y' primers, 25 to 40 cycles under the conditions for the first PCR.

Each PCR can be started with an initial denaturing and ended with a concluding extension under the respective aforementioned conditions.

The amplified cytokeratin cDNA is detected in the usual manner, e.g., via agarose gel electrophoresis with ethidium bromide under UV light or southern blot hybridization with the probes specific for the cytokeratin cDNA.

Another subject of this invention is a kit for detecting CK18 in a body sample. This kit includes means for converting mRNA into cDNA, primers for a PCR, reagents, solutions, buffers and enzymes, along with means for detecting amplified DNA.

The primers in the kit are the above X, Y and/or X',Y' primers.

This invention offers several advantages. The slightest amounts of CK18 RNA can be detected with the method according to the invention. For example, the detectable amounts of CK18 RNA make it possible to discern 1-10 epithelial cells per milliliter of blood. The method according to the invention is hence very sensitive. In addition, it yields no false-positive results, which also makes it very specific. Therefore, the method according to the invention is highly suited for detecting CK18.

Brief Description of Drawings

Fig. 1 shows the cDNA of CK18 and the DNA of three processed CK18 pseudogenes. The positions of primers X, Y, X' and Y' are also indicated in the CK18 cDNA.

Fig. 2 shows a partial range of the CK18 cDNA and corresponding partial ranges for three processed CK18 pseudogenes, as well as the construction of "upper" and "lower" primers, wherein the nucleotide substitutions are depicted in boxes.

Fig. 3 shows amplified cDNA's of a PCR reaction (expected size of amplified cDNA: 210 bp) during gel electrophoresis with ethidium bromide dye (0-5 : 10^0 to 10^5 HT29 carcinoma cells per milliliter of peripheral blood, N: negative control (water instead of blood during RNA isolation, H: negative control (water instead of RNA during cDNA synthesis, B: peripheral blood without carcinoma cells).

Fig. 4 shows the study of bone marrow samples using a method according to the invention (ethidium bromide-dyed gel, H: negative control (water instead of RNA during cDNA synthesis), P: positive control (RNA of carcinoma cells in peripheral blood); 1: bone marrow sample of a patient with nonmalignant disease (chronic pancreatitis); 2-6: bone marrow samples of patients with gastrointestinal cancer.

The following examples illustrate the invention.

Example 1: Cloning of processed CK18 pseudogenes

Genomic DNA was isolated from leukocytes of the peripheral blood according to a conventional method and treated with 30 µg/ml of RNase for one hour at 37°C. A PCR was performed, using the following primers to amplify CK18 pseudogenes: L: 5'-ATGAGATTCACCACTCGCTCCACCT-3' and R: 5'-ATGCCTCAGAACTTTGGTGTCATTGG-3'. The PCR reaction conditions involved 32 cycles at 97°C (1 minute), a hybridization temperature of 62°C (2 minutes) and a concluding extension step at 72°C (2 minutes), followed by an extension at 72°C (10 minutes). The PCR batch consisted of 50 mM KCl, 10 mM tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatins, 0.25 mM of each dNTP, 0.4 µM of primer and 5 units of Taq polymerase per 100 µl. The amplified cDNA was integrated into the cloning point of the pCRII vector. The obtained clones were subjected to a restriction analysis, revealing three clones containing the CK18 pseudogenes shown on Fig. 1.

Example 2: Detection of CK18 in a method according to the invention

Cell cultures of the human colon carcinoma cell line HT29 were treated with trypsin, pelleted, washed, resuspended in PBS, counted and serially diluted in the peripheral blood of healthy individuals containing 153 mg of hemoglobin and 6 x 10^6 leukocytes per milliliter of blood (10^5 to 10^0). While collecting blood samples, care was taken to prevent epithelial cells from contaminating the skin by discarding the first milliliters of sampled blood. RNA was extracted from the blood or blood-HT29 dilutions in a conventional manner using the guanidinium-thiocyanate-phenol-chloroform method. In addition, the RNA preparation was incubated with DNase (40 units per 400 µl with 10 units of RNase inhibitor in 5 mM of $MgSO_4$ at 25°C). The enzyme was then inactivated at 90°C for 5 minutes. Dried RNA of 0.5 ml of blood or blood-HT29 dilutions was resuspended in 80µl of TE. This RNA was subjected to reverse transcription using the "random hexamer" primer. This yielded cDNA. This was subjected to a "nested" PCR, in which "nested" primers were used as the second primer pair. The first PCR batch (100 µl) consisted of 10 µl of cDNA and a respective 0.8n µM of the primers 5'-

TGCTCACCACACAGTCTGAT-3' (X) and 5'-CACTTTGCCATCCACTAGCC-3' (Y). 40 cycles were executed at 95°C (50 seconds) and 54°C (30 seconds). 7 µl of this batch were added to a second PCR batch (100 µl), which contained a respective 0.8 µM of the primers 5'-TGGAGGACCGCTACGCCCTA-3' (X') and 5'-CCAAGGCATCACCAAGACTA-3' (Y''). In addition, each PCR was started with an initial denaturing for 80 seconds at 95°C, and ended with a concluding extension for 1 minute at 72°C. Amplified cDNA was subjected to gel electrophoresis to estimate the size and amount of amplified cDNA.

As evident from Fig. 3, the amounts of CK18 detected in the peripheral blood made it possible to discern 1-10 cells of the colon carcinoma cell line HT29 per milliliter of blood. As also evident from Fig. 3, no CK18 could be detected in the blood of healthy individuals without adding epithelial cells.

The method according to the invention is hence specific and sensitive relative to cytokeratins, e.g., CK18.

Example 3: Detection of CK18 in patient samples

Bone marrow samples were taken from five patients suffering from esophageal, stomach and lung tumors and one patient with a benign illness (chronic pancreatitis). A method according to the invention was executed (compare Example 2).

As evident from Fig. 4, three of the five tumor patient samples tested positive for CK18. The sample from the patient with chronic pancreatitis was negative for CK18.

The method according to the invention can hence be used to specifically, i.e., without any false-positive results caused by genomic DNA, and sensitively detect cytokeratin, e.g., CK18, in patient samples.

Claims

1. Oligonucleotides of the following sequence: 5'-TGCTCACCACACAGTCTGAT-3' (X), 5'-CACTTTGCCATCCACTAGCC-3' (Y), 5'-TGGAGGACCGCTACGCCCTA-3' (X'), 5'-CCAAGGCATCACCAAGACTA-3' (Y').

2. Application of the oligonucleotides according to Claim 1 as primers for detecting cytokeratin 18 (CK18).

3. Method for detecting cytokeratin 18 (CK18), involving the following steps:

a) Taking and preparing a body sample,

b) Isolating mRNA from the body sample,

c) Converting the mRNA into cDNA through reverse transcription,

d) Amplifying the cDNA using a PCR with primers exhibiting a higher affinity to the cDNA of CK18 than to processed CK18 pseudogenes, and

e) Detecting the amplified cDNA,

characterized in that the primers are 5'-TGCTCACCACACAGTCTGAT-3' (X), 5'-CACTTTGCCATCCACTAGCC-3' (Y) and/or 5'-TGGAGGACCGCTACGCCCTA-3' (X'), 5'-CCAAGGCATCACCAAGACTA-3' (Y').

4. Method according to Claim 3, characterized in that the body sample is a smear, the punctate fluid or biopsy of an organ, e.g., bone marrow, but also blood, sputum, urine, stool, fluid, bile, lymphatic liquid or gastrointestinal secretion.

5. Method according to Claim 3 or 4, characterized in that the cytokeratin pseudogenes are those from Fig. 1.

6. Kit for detecting cytokeratin 18 (CK18), consisting of means for converting mRNA into cDNA, primers for a PCR, reagents, solutions, buffers and enzymes, along with means for detecting amplified cDNA, characterized in that the primers are 5'-TGCTCACCACACAGTCTGAT-3' (X), 5'-CACTTTGCCATCCACTAGCC-3' (Y) and/or 5'-TGGAGGACCGCTACGCCCTA-3' (X'), 5'-CCAAGGCATCACCAAGACTA-3' (Y').

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5 page(s) of drawings attached
